### Remarks

In response to Applicants' interview with the Examiner on April 3, 2008, and in response to the Examiner's email correspondence of June 11, 2008, Applicants respectfully request that the present *Supplemental Amendment and Reply* be entered and considered (along with the *Amendment and Reply* and other documents submitted by Applicants on March 5, 2008) in response to the Office Action mailed Oct. 5, 2007.

Applicants respectfully submit that the claims presented herein should be allowed based on the specification and claims as originally filed and based on the explanations, arguments, evidence and affidavits previously submitted in replies to office actions in the present application. New claim 97 is submitted herein as a follow-up to Applicants' interview with the Examiner on April 3, 2008 and in response to the Examiner's email correspondence of June 11, 2008. In particular, during an April 3, 2008 interview with the Examiner, the Examiner agreed to consider the allowability of a product-by-process claim in response to the currently pending office action in the present application. Additionally, in a June 11, 2008 email from the Examiner, the Examiner requested that Applicants submit a *formal* amendment in place of the informally proposed claim amendment previously submitted for the Examiner's consideration on April 30, 2008. Additionally, the Examiner's June 11<sup>th</sup> email message requested "a discussion on why this claim should be allowed." Accordingly, Applicants submit herewith a formal *Supplemental Amendment and Reply* to enter the previously proposed product-by-process claim (new claim 97) along with a discussion on why this claim should be allowed.

## Allowance of New Product-By-Process Claim 97

Applicants note that new claim 97 finds support in the present application as originally filed. For example, support for claim 97 can be found in the specification at: page 11, line 12 to page 12, line 5; and, Examples 1 through 4, page 25 to page 30.

Furthermore, Applicants submit that new claim 97 should be allowed because the prior art does not teach or suggest that a combination of:

4

- 1) incubating human bone marrow cells under a low oxygen condition such that said cells form adherent cell colonies (CFU); and,
- 2) passaging the adherent cell colonies obtained at a seeding density of less than about 2500 cells/cm<sup>2</sup>

would produce an isolated population of human bone marrow cells wherein greater than about 91% of said passaged cells co-express CD49c and CD90, and wherein said passaged cells maintain a population doubling rate of less than about 30 hours after 30 cell doublings.

Indeed, the prior art does not teach or suggest that applying a combination of low oxygen concentration and low cell density passaging to the isolation and culturing of human bone marrow cells would produce the unique population of cells that Applicants have obtained via the process recited in claim 97. In other words, obtaining the presently claimed unique, isolated population of human bone marrow cells by combining low oxygen conditions and low cell density culturing is a novel, as well as a surprising and unexpected result.

Hence, Applicants particularly submit that no publications previously cited by the Examiner<sup>1</sup> nor any prior art of which Applicants are currently aware teaches or suggests isolation of, or the possibility of isolating, the unique cell population of claim 97. Moreover, the prior art does not teach, suggest, or motivate one of skill in the art to try to obtain, or to expect that one could obtain, the presently claimed unique cell population via a combination of low oxygen condition and low cell density passaging as recited in claim 97. In particular, Applicants note that the human bone marrow cell population so obtained is one wherein the cells express, *inter alia*, CD13, CD44, CD49c, CD90, HLA Class-1 and β (beta) 2-Microglobulin, but do not express, *inter alia*, CD10, CD34, CD45, CD62L, or CD106. *See*, Specification as originally filed as well as Exhibits and Declarations Under 35 U.S.C. § 132 submitted in the present application on May 18, 2007 and March 5, 2008. None of the cells of the prior art have all of these characteristics. These characteristics, while not mentioned in the claims, are inherent in the cells prepared by

<sup>1</sup> See, for example: Haynesworth et al., U.S. Patent 5,733,542; Pittenger et al., Science 284:143-147 (1999); Woodbury et al., J. Neurosci. Res. 61: 364-370 (2000); Lee et al., Hepatology 40: 1275-1284 (2004); Jiang et al., Nature 418:41-49 (2002); Furcht et al., U.S. Patent 7,015,037; Caplan et al., U.S. Patent 5,486,359; Caplan et al., U.S. Patents 5,811,094; and, Caplan et al., U.S. Patent 5,837,539.

the steps that are claimed in claim 97. Accordingly, Applicants respectfully submit that the product-by-process claim of new claim 97 is fully supported by the specification as originally filed and should be allowed in view of the prior art.

## "Seeding Density"

On a different but currently pertinent topic, Applicants note that new claim 97 refers to a cell "seeding density" of less than about 2500 cells/cm<sup>2</sup>. In this regard, the Examiner recently issued a rejection in one of Applicants' related patent applications<sup>2</sup>, wherein the phrase "seeding density of less than about 30 cell/cm<sup>2</sup>" was rejected as allegedly indefinite under 35 U.S.C. § 112, second paragraph. See, Paper No. 20080414, page 2, last paragraph (mailed 05/23/2008 in U.S. Application No. 11/054,824). In particular, the Examiner stated:

The limitation "seeding density of less than about 30 cell/cm<sup>2</sup>" renders the claims indefinite because density must be measured as mass/volume and the cited phrase is "\_\_/area." Therefore, the amount of cells to be used in the instant invention is unclear and the intended claim coverage is indefinite.

*Id* (emphasis added).

Applicants respectfully disagree and traverse this rejection on the basis that "seeding density" is, indeed, properly defined as *per unit area*. See, e.g., The New College Edition of The American Heritage Dictionary of the English Language, Ed. William Morris, Houghton Mifflin Co., Boston, MA, p. 353 (1976) which defines "density" as:

- 1. The degree or a measure of the degree to which anything is filled or occupied.
- 2. Physics.
  - a) The amount of something per unit measure, especially per unit length, area, or volume.
  - b) The mass per unit volume of a substance under specified or standard conditions of pressure and temperature. Also called "mass density."
- 3. The number of inhabitants per unit geographical region. Also called "population density".

<sup>&</sup>lt;sup>2</sup> See, U.S. Application No. 11/054,824.

See, Exhibit A (emphasis added).

Based on the above-cited rejection, however, the Examiner indicated that to meet the requirements of 35 U.S.C. § 112, second paragraph, new claim 97 must define "density" according to the Examiner's particularly selected definition (i.e., "mass density") which is one of at least seven possible definitions (three of which are shown above; i.e., definition 2.b). See, Exhibit A. In contrast, it is, and would be, readily apparent to those of ordinary skill in the art that the presently pending specification (and new claim 97) refers to "cell density" and "seeding density" in a manner consistent with commonly understood definitions 1. or 2.a) from the above-referenced dictionary. Furthermore, Applicants submit herewith Exhibits B through G to show that "cell density" and "seeding density" specified as a number of cells per unit area (e.g., cells/cm<sup>2</sup>) is a term that has long been used, and is readily understood, by those in the field of cell biology. In particular, Exhibits B through G show abstracts from a sampling of six publications ranging from 1981 to 2000 wherein cell density is specified according to the number of cells seeded per unit area (i.e., per/cm<sup>2</sup>). Finally, the specification as originally filed also teaches that "A seeding density would be the number of adherent cells per cm<sup>2</sup> obtained from mononuclear bone marrow cells." Specification, page 12, lines 8-9.

Thus, with respect to use of the term "seeding density" in claim 97, Applicants submit that this term is used in a manner consistent with a commonly used dictionary definition, in a manner routinely understood by those of ordinary skill in the art, and in a manner consistent with the definition provided in the specification as originally filed. Therefore, Applicants submit that a rejection of new claim 97 under 35 U.S.C. § 112, second paragraph, would be improper.

## Application Pendency

Applicants also note that the present application has now been pending for almost seven years. As such, Applicants point out that in situations such as this the Examiner and the Examiner's Supervisor are to consider the application "special" and to expedite prosecution to a final disposition:

# 707.02 Applications Up for Third Action and 5-Year Applications

The supervisory patent examiners should impress their assistants with the fact that the shortest path to the final disposition of an application is by finding the best references on the first search and carefully applying them.

The supervisory patent examiners are expected to personally check on the pendency of every application, which is up for the third or subsequent Office action with a view to finally concluding its prosecution.

Any application that has been pending five years should be carefully studied by the supervisory patent examiner and every effort should be made to terminate its prosecution. In order to accomplish this result, the application is to be considered "special" by the examiner.

See, M.P.E.P. § 707.2, Eighth Ed., Rev-d Sept. 2007.

Based on the foregoing Supplemental Amendment and Reply and the Amendment and Reply submitted on March 5, 2008 in response to the currently pending Office Action (as well as all previously filed responses, explanations, arguments, evidence, and affidavits) Applicants respectfully request that all outstanding rejections and objections be withdrawn and that previously amended claims 14, 21, 25, 26 and new claim 97 be considered and passed to allowance.

## Conclusion

All of the stated grounds of rejection and objection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Reply is respectfully requested.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX

P.L.L.C.

Doyle A. Siever Attorney for Applicants Registration No. 47,088

Date:

1100 New York Avenue, N.W. Washington, D.C. 20005-3934 (202) 371-2600

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# THE AMERICAN HERITAGE DICTIONARY OF THE ENGLISH LANGUAGE

# THE AMERICAN HERITAGE ON THE AMERICAN HERITAGE ON THE AMERICAN HERITAGE

OF THE ENGLISH LANGUAGE

WILLIAM MORRIS, Editor

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lent to ten bronze asses, ued at 25 silver denarii, ss, from adjective, "conee dekm- in Appendix.\*]
2. Divided or counted by ienarius.]

tr.v. -ized, -izing, -izes, i, or characteristics. 2. To notion) to private owner-

: -Ized, -Izing, -Izes. 1. To rights of naturalization or

nuring, -tures, Aiso de-na. I. To change the nature or lit to eat or drink; espedechol for this purpose, atter to (fissionable mate de-na'tur-ant n

ade unfit for drinking by methanol. Hre (děn'bě-shîr, -shər). A i of northeastern Wales.

Having the characteristic Having the characteristic:
(i)- + -FORM.]
al crystallizing in another
r treelike mark. b. A rock
arks. 2. A branched part
s toward the cell body. In
DENDR(O)- + -ITE.]
n-drit-i-eal (-I-kal). 1. Of,
e. 2. Tree-shaped; dendri-

...

s tree; for example, den-Latin, from Greek, from

nol'a-je) n. The study of ine and date past events.

ides: DBNDR(O)- + -OID.]
botanical study of trees. : (-dra-lŏj'lk), den'dro-log'-

star in the constellation ears from Earth. [Arabic

cond-brightest star in the al-(asad), "tail of the

denial. [Middle English tion, from Latin denegatio,

negare, DENY.] demic disease transmitted y fever, rash, and severe eakbone fever," "dandy." Swahili kidinga.]
of being denied; question-

reply, as to a request; a isal to grant the truth of a in. 3. A rejection, as of a or disavowal; repudiation.

. -ized, -izing, -izes. Also -tining, -tines. To remove

es.

1se 1: also do-nir for sense
on, nylon, and silk yarns,
as per 450 meters of yarn.
sition and value current in
the eighth century until the nall or trifling sum. [Mid-m Old French denier, from

d, -grating, -grates. a, -graung, -graves. 1 to or reputation of; defame. 

zre, to blacken: dē-, comniger, black (see niger in den'i-gra'tor (-gra'tor) n. 
illed cloth used for overalls ents made of coarse denim. draperies and upholstery.

L Also Den.ys. Apostle to irst Bishop of Paris; patron

-fying, -fies. To remove al compound), as by bacte-

tion n. bitant; resident. 2. British. s and privileges of citizen-t naturalized in a region to denizened, -zening, -zens.

r pler/j judge/k kick/l lid, roar/s sauce/sh ship, dish/

flish. To make a denizen of: naturalize. [Middle English misch, from Old French denzeln, from deliz, within, from law Latin delinus, from within: Latin de. from + intus, within of an in Appendix's). —den't-zen-a'tion.

Gri-mark (den'mark'). Danish Dan-mark (dan'mark'). Abbr. Danish Can'mark'). Abbr. Danish Dan-mark (dan'mark'). Abbr. Danish Danish (dan'mark'). Abbr. Dan

pagen.

Def-mark Strait (den'märk'). A sea passage, 300 miles long and 130 miles wide, between Greenland and Iceland, connecting the Arctic Ocean with the Atlantic.

den mother. A woman who supervises a den of Cub Scouts.

Def-ner (dén'er). Johann Christoph. 1655-1707. German maker of woodwind instruments; inventor of the clarinet.

Tearnia (dén'is). Also Danie. A measuline clarinet.

Den-nls (dën'is). Also Den-le. A masculine given name. [Mid-die English Denes, Dionisius, from Latin Dionysius, Dionysus.]

de English Densis Diomisias, from Lain Diomysia, Diorvsts, J de-nom-i-nate (id-nōm'o-nāt') /r.v. -nated, -nating, -nates. To give a name to; designate. [Lain denomināre : de-, completely + nōmināre, to name, from nōmen, name (see nomen- in Appendix\*). —de-nom'i-na-bis (-na-boi) ad/. de-nom-i-nate number (id-nōm'o-nīt). A number that desig-nates a quantity as a multiple of a unit. In the expression 12 named 12 is a denominate number.

nates a quantity as a multiple of a unit. In the expression 12 pounds, 12 is a denominate number.

de-nom-i-na-tion (dl-nom's-nā'shən) n. 1. The act of naming.

2. A name; designation. 3. The name of a class or group; classification. 4. A class of units having specified values, as in a system of currency or weights. 5. Abbr. denom. An organized group of religious congregations. —See Synonyms at name.

-de-nom-i-na-tion-al-iam (dl-nom's-nā'shən-ə-liz'am) n. 1. The tendency to separate into religious sects or denominations.

2. Advocacy of such separation. 3. Strict adherence to a denomination; sectarianism. —de-nom'i-na'tion-al-iat n. de-nom'i-nation-al-iat n. Giving or

normation; sectarianism. — se-norm than unnation is de-norm-in-attive (di-norm-in-attive). Giving or constituting a name; naming; appellative. 2. Grammar. Formed from a houn or adjective. — m. A word, especially a verb, that is derived from a noun or adjective, such as the verb to bus from the noun bus.

the noun bus.

de-noin-i-na-tor (di-nom's-na'tor) n. The quantity below the line indicating division in a fraction; the quantity that divides the numerator. Compare numerator.

de-no-ta-tion (de'no-ta'shon) n. 1. The act of denoting; indication.

2. A sign, symbol, or reference that denotes; indicator.

3. Something signified or referred to; a particular meaning of a symbol.

4. The explicit meaning of a word, as opposed to its connotation.

symbol. 4. The explicit meaning of a word, as opposed to its connotation. 
de-not-ative (di-nō'tɔ-ulv, dē'nō-tā'-) adj. 1. Able to denote; 
designative. 2. Explicit. —de-no'ta-tive-ly adv. 
de-note (di-nōt') tr.v. -noted, -noting, -notes. 1. To reveal or 
indicate; mark. 2. To serve as a symbol or name for; signify. 
3. To refer to specifically; mean explicity. —See Synonyms at 
mean (convey sense). [Old French denoter, from Latin dênotare: dē-, completely + notare, mark, from nota, NOTE.] —de-not'a-ble adj. —de-not'avle adj. 
dé-nous-ment (dā-nōō-mān') n. Also de-nous-ment. 1. The 
solution, clarification, or unraveling of the plot of a play or 
novel. 2. Any outcome or final solution. [French, "an untying," from Old French desnouement, from desnotujer, undo 
ide-nounce (di-nouns') tr.v. -nounced, -nouncing. -nounces. 
1. To condemn openly; censure, especially as evil. 2. To accuse 
formally; inform against. 3. To give formal announcement of 
the ending of (a treaty). —See Synonyms at critelize. [Middle 
English denouncen, from Old French denoncier, announce, from 
Latin dêminitare, make an official announcement of : dē-, 
completely + minitare, announce, from minitus, messenger (see 
nou-1 in Appendix's). ] —de-nounce'ment n. —de-nounce' nr. 
dense (dēns) adj. denser, densest. 1. a. Having relatively high 
density. b. Crowded closely together; compact. 2. Thick; impenetrable. 3. Thickheaded; dull. 4. Photography. Opaque, 
with good contrast between light and dark areas. Said of a 
developed negative, —See Synohyms at stupid. [Latin dēnsus. 
See dens-2 in Appendix.'] —dense'nes n. 
den-sime-ter (dēn-sim'o-ter) n. An instrument used to determine density. [Latin dēnsus, Dense + Meter.] —dense'nese n. 
den-sime-ter (dēn-sim'o-ter) n. An instrument used to determine density. [Latin dēnsus, Dense + Meter.] —dense'nese n. 
den-sime-ter (dēn-sim'o-ter) n. An instrument used to determine density. [Latin dēnsus, Dense + Meter.] —dense'nese n. 
dens-sime-ter (dēn-sim'o-ter) n. An instrument used to deter-

mine density. [Latin densus, DENSE + METER.]—den'el-met'-ric (-s-mēt'rīk) adi. densi-tom-e-ter (den's-tom'o-tor) n. An apparatus for measuring the optical density of a material, such as a negative.

measuring the optical density of a material, such as a negative. [DENSITY) + METER.]
Gen-si-ty (ddn'ss-tè) n. pl. ties. 1. The degree or a measure of the degree to which anything is filled or occupied. 2. Physics.

The amount of something per unit measure, especially per unit length, area, or volume. See charge density, current density, energy density. b. The mass per unit volume of a substance under specified or standard conditions of pressure and temperature. Also called "mass density." 3. The number of inhabitants per unit geographical region. Also called "population density."

4. The degree of optical opacity of a medium or material, as of a photographic negative. 5. Thickness of consistency; impenetrability. 6. Stupidity; dullness.

Gent (dent) n. A depression in a surface made by pressure or a blow.—». dented, denting, dents.—tr. To make a dent in.—Intr. To become dented. [Middle English dent, variant of dunt, strike, blow. Old English dynt, from Germanic duntifunattested).]

(unattested).]
dent. dental; dentist; dentistry.

den-tal (děnt\*l) adj. 1. Abbr. dent. Of, pertaining to, or for the teeth. 2. Abbr. dent. Of, pertaining to, or for dentistry. 3. Phonetics. Produced with the tip of the tongue near or against the upper front teeth; alveolar. —n. Phonetics. A dental consonant. [New Latin dentalis, from Latin dens (stem dent-), tooth. See dent- in Appendix. dental floss. A strong waxed thread used to clean areas between the teeth.

between the teeth. denture (see). dental plate. A denture (see). dental plate. A denture (see). dental plate. A denture (see). Edged with toothlike projections; toothed. [Latin dentātus, from dēns (stem dent-), tooth. See dent- in Appendix. ]—den'tate'ly adv. den-ta-tion (dēn-tā'shən) n. 1. The condition of being dentale. 2. A toothlike part or projection. dent oorn. A tall-growing variety of corn, Zea mays indentala, having yellow or white kernels that are indented at the tip. denti-, dent-. Indicates tooth; for example, dentiform, dentold. [From Latin dēns (stem dent-), tooth. See dent- in Appendix. ]

told. [From Latin dens (stem dent-), tooth. See gent in appendix.\*] den-ti-cle (den'ti-kel) n. A small tooth or toothlike projection. [Middle English, from Latin denticulus, diminutive of dens (stem dent-), tooth. See dent- in Appendix.\*] den-tic-u-late (den-tik-ye-lit) adj. Also den-tic-u-late-ed (-la'tid) 1. Finely toothed; minutely dentate. 2. Architecture. Having dentils. [Latin denticulatus, from denticulus, DENTICLE.] —den-tic-u-late-y adv. —den-tic

-FORM.] den-ti-frice (den'to-fris) n. A substance, such as a powder or paste, for cleaning the teeth. [Old French, from Latin den-tifricium: DENTI- + fricāre, to rub (see bhrēi- in Appendix').] den-til (den'til) n. Architecture. One of a series of small rectangular blocks forming a molding or projecting beneath a cornice. [Obsolete French dentile, from Old French, diminutive of dent, tooth, from Latin dens (stem dent-), tooth. See

utive of dent, tooth, from Latin dens (stem dent-), tooth. See denti-] denti-la-bi-al (dën'ti-la'bē-al) adj. Phonetics. Labiodental. [DENTI-+ LABIAL.] —den'ti-la'bi-al n. den-ti-lin-gual (dën'ti-ling'gwal) adj. Phonetics. Interdental. [DENTI-+ LINGUAL.] —den'ti-ling gual n. den-ti-lin-gual (dën'ti-ling'gwal) adj. Phonetics. Interdental. [DENTI-+ LINGUAL.] —den'ti-ling gual n. den-tine (dën'tën') n. Also den-tin (-tin). The calcareous part of a tooth, beneath the enamel, containing the pulp chamber and root canals. [DENTI()-+ -INE.] —den'ti-nal adj. den-tist (dën'tist) n. Abbr. dent. A person whose profession is dentistry. [French dentiste, from dent, tooth. See dentil.] den-tist-ry (dën'ti-strè) n. Abbr. dent. The diagnosis, prevention, and treatment of diseases of the teeth and related structures, including the repair or replacement of defective teeth. den-ti-tion (dën-tish'ən) n. 1. Biology. The type, number, and arrangement of teeth, especially in animals. 2. The cutting of teeth; a teething: [Latin dentitio, from dentitus, past participle of dentire, teethe, from dens (stem den-), tooth. See dentil.] den-told (dën'toid') adj. Toothiike. [DENTI()-+-0D.]
D'En-tre-cas-teaux Islands (dän'tre-kas'tō'). An island group, about 1,200 square miles in area, in the southwestern Pacific, administered as part of the Trust Territory of New Guinea. den-tu-lous (dën'char) n. A set of artificial teeth. Also called "dental plate." [French, from Old French, from dent, tooth. See dentil.]
de-nu-date (di-nōò'dät', di-nyōò'-) tr.v. -dated, -dating, -dates. To denude. —adl. Bare: denuded. [Latin dendatare. DENUME.]

"dental plate." [French, from Old French, from dent, tooth. See dentil.]
See dentil.]
To denude. —adi. Bare; denuded. [Latin dênidâre, DENUDE.]
denude (di-nōō' dāt', di-nyōō') tr.v. -dated, -dating, -dates.
To denude. —adi. Bare; denuded. [Latin dênidâre, DENUDE.]
denude (di-nōō', nyōōd') tr.v. nuded, -nuding, -nudes. 1. To
divest of covering; make bare. 2. Geology. To expose (rock
strata) by erosion. —See Synonyms at etrip. [Latin denidâre:
dē-, thoroughly + nūdāre, to make bare, from nūdus, NUDE.]
—den'u-da'tion (dēn'yōō-dā'shan)n.
de-nu-ner-a-ble (di-nōō'ms-a-be), di-nyōō'-) adj. Capable of
being put into one-to-one correspondence with the positive
integers; countable. —de-nu'mer-a-bly adv.
de-nun-ci-ate (di-nūn'sō-ā'shan, -shō-ā'shan)n. 1. The act of
denun-ci-a-tion (di-nūn'sō-ā'shan, -shō-ā'shan)n. 1. The act of
d

say no (see no in Appendix?).]

Synonyms: deny, gainsay, contradict, refute. These verbs mean to dispute the truthiluness of a statement or speaker. Deny, the most general, usually implies an open declaration that something is untrue. Gainsay more often appears in the negative to stress the unlikelihood or impossibility of opposing or rejecting. Contradict involves an assertion that the opposite of a statement is the Bailton making in the markelihou of suidence it disc is true. Refute emphasizes the marshaling of evidence to dis-

is true. Refute emphasizes the management of order of prove something.

Denrys. See Saint Denis.

de-o-dar (de'o-dar') n. A tall cedar, Cedrus deodara, native to the Himalayas, having drooping branches and wood valued as timber. [Hindi de'odār, from Sanskrit devadāru: devās, divine (see delw- in Appendix\*).]



t tight/th thin, path/lh this, bathe/ü cut/ür urge/v valve/w with/y yes/z zebra, size/zh vision/e about, item, edible, gallop, circus/a Fr. ami/ce Fr. feu, Ger. schön/ü Fr. tu, Ger. über/kH Ger. ich, Scot. loch/N Fr. bon. \*Follows main vocabulary. †Of obscure origin.

Transfusion, 40(11):1299-307 (2000).

Serum supplement, inoculum cell density, and accessory cell effects are dependent on the cytokine combination selected to expand human HPCs ex vivo.

Xu R, Medchill M, Reems JA.

BACKGROUND: The prolonged periods of pancytopenia associated with cord blood transplants suggest that in some cases cell numbers may be limiting. The possibility that limiting cell numbers may be overcome and prolonged periods of pancytopenia abrogated by the transplantation of human umbilical cord blood cells expanded ex vivo has led to efforts to define optimal culture conditions for these cells. STUDY DESIGN AND METHODS: Cord blood CD34+ cells were cultured with three cytokine combinations: SCF+G-CSF+GM-CSF+MGDF SCF+MGDF+Flt3-ligand (6SMF); and IL-1+IL-3+IL-6+G-CSF+GM-(SGGM); IL-6+ CSF+SCF+Epo (GFmix). Serum effects, inoculum concentration (cells/mL) seeding density (cell/cm(2)) and accessory cell effects on the expansion of CD34+ cells were determined. RESULTS: Cellular outputs were significantly higher with fetal calf serum (FCS) than with cord blood serum (CBS) or adult group AB serum (ABS) in the presence of 6SMF, however, CBS was as effective as FCS. The best seeding concentrations varied for each of the cytokine combinations, and inoculum densities exceeding 1000 cells per cm(2) proved detrimental for cultures containing GFmix and SGGM. Accessory cell studies indicated that populations expressing the CD33 antigen inhibited the expansion of purified CD34+ cells in the presence of GFmix or SGGM, but not in the presence of 6SMF. CONCLUSION: Serum supplement, inoculum cell concentration, seeding densities, and accessory cell effects are dependent upon the cytokine combination selected to expand cord blood HPCs ex vivo. Thus, each of these measures should be assessed to establish reproducible and reliable conditions for the selection of different cytokine combinations to culture cord blood HPCs.

Biomaterials, 21(12):1207-13 (2000)

Marrow stromal osteoblast function on a poly(propylene fumarate)/beta-tricalcium phosphate biodegradable orthopaedic composite.

## Peter SJ, Lu L, Kim DJ, Mikos AG.

The objective of this study was to assess the osteoconductivity of a poly(propylene fumarate)/beta-tricalcium phosphate (PPF/beta-TCP) composite in vitro. We examined whether primary rat marrow stromal cells would attach, proliferate, and express differentiated osteoblastic function when seeded on PPF/beta-TCP substrates. Attachment studies showed that a confluent monolayer of cells had adhered to the substrates within an 8 h time frame for marrow stromal cells seeded at confluent numbers. Proliferation and differentiated function of the cells were then investigated for a period of 4 weeks for an initial seeding density of 42,000 cells/cm2. Rapid proliferation during the first 24 h as determined by 3H-thymidine incorporation was mirrored by an initial rapid increase in total cell number by DNA assay. A lower proliferation rate and a gradual increase in cell number persisted for the remainder of the study, resulting in a final cell number of 128,000 cells/cm2. Differentiated cell function was assessed by measuring alkaline phosphatase (ALP) activity and osteocalcin (OC) production throughout the time course. Both markers of osteoblastic differentiation increased significantly over a 4-week period. By day 28, cells grown on PPF/beta-TCP reached a maximal ALP activity of 11 (+/- 1) x 10(-7) micromol/min/cell, while the OC production reached 40 (+/- 1) x 10(-6) ng/cell. These data show that a PPF/beta-TCP composite exhibits in vitro osteoconductivity similar to or better than that of control tissue culture polystyrene.

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## Nicotine transport in a human choriocarcinoma cell line (JAR).

Zevin S, Schaner ME, Giacomini KM.

Smoking is a major health problem in pregnancy resulting in intrauterine growth retardation and birth complications. Nicotine, a toxic component of cigarette smoke, interferes with amino acid transport in the placenta and stimulates catecholamine release resulting in uteroplacental vasoconstriction. Transplacental transport of nicotine may be an important determinant of placental and fetal exposure. Our aim was to determine the mechanism of nicotine transport in the human choriocarcinoma cell line, JAR, as a model for the placenta. JAR cells were subcultured in 12-well plates following trypsinization at a seeding density of 0.5 x 10(6) cells/well (1.3 x 10(5) cells/cm2). Uptake studies of [3H]nicotine were carried out in JAR cell monolayers on day 2 after plating. [3H]Nicotine uptake was saturable (Km 156 microM), sensitive to temperature, and inhibited by unlabeled nicotine and various organic cations including mecamylamine and quinidine, but not by guanidine, tetraethylammonium (TEA), or neurotransmitters. Counterflux of [3H]nicotine uptake was produced by unlabeled nicotine and mecamylamine but not by cotinine or acetylcholine, consistent with a carrier-mediated transport process. The uptake could be driven by an inside-negative membrane potential or by an outwardly directed pH gradient. This is the first demonstration of a carrier-mediated transport mechanism for nicotine in a human cell line. This transport mechanism may have implications to the disposition of nicotine in the human placenta.

Exp. Cell. Res., 194(2):186-9 (1991)

Density-related expression of caldesmon and vinculin in cultured rabbit aortic smooth muscle cells.

Shirinsky VP, Birukov KG, Koteliansky VE, Glukhova MA, Spanidis E, Rogers JD, Campbell JH, Campbell GR.

Quantitative immunoblotting techniques were used to study the effects of seeding density on the expression of caldesmon and vinculin variants, which are sensitive markers of vascular smooth muscle cell (SMC) phenotypic modulation in culture. Rabbit aortic SMC were seeded at different densities: 13 x 10(4) cells/cm2 (high density), 3 x 10(4) cells/cm2 (medium density), and 0.2 x 10(4) cells/cm2 (low density) and cultured in the presence of 5% fetal calf serum. Irrespective of cell density and growth phase, caldesmon150 was gradually and irreversibly substituted by caldesmon77, but at high seeding density this substitution proceeded at a slower rate. The fraction of meta-vinculin (smooth muscle variant of vinculin) was reduced after seeding SMC in culture, but was reestablished when the cells reached confluency. Thus, high SMC seeding density is essential but not sufficient to keep vascular SMC cultured in the presence of serum in the contractile phenotype.

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## Effect of seeding density and time in culture on vascular smooth muscle cell proteins.

Seidel CL, White V, Wallace C, Amann J, Dennison D, Schildmeyer LA, Vu B, Allen JC, Navarro L, Eskin S.

The purpose of this work was to determine the effect of seeding density and time in culture on quantitative and qualitative characteristics of myosin in primary cultures of vascular smooth muscle cells (VSMCs). Enzymatically dispersed VSMCs from femoral arteries and saphenous veins of adult dogs were seeded at a density of 10(3)-10(5) cells/cm2 and assayed after 7 days or at 10(5) cells/cm2 and assayed between 1 and 10 days. Myosin, actin, and total protein contents as well as electrophoretic and immunoreactive characteristics of myosin heavy chains (MHCs) were determined. Total and contractile protein contents were independent of seeding density and increased with time in culture. Freshly dispersed cells exhibited two MHCs (MHC-1 and MHC-2) but, within 24 h after culturing, only cells attached to the dish expressed a third protein band (MHC-3), which had electrophoretic mobility and immunoreactivity similar to purified platelet MHC. MHC-3 appeared before onset of cell division and, by 4 days in culture when cells were proliferating, became the dominant MHC form. Loss of MHC-1 and MHC-2 could be prevented by growing cells in a serum-free, defined media that prevented proliferation. These data indicate that seeding density does not affect myosin content, but that with time in culture expression of a MHC with characteristics similar to nonmuscle myosin occurs. Expression of MHC-3 is associated with cell attachment, whereas loss of MHC-1 and MHC-2 requires proliferation.

*In Vitro*, 17(8):713-8 (1981).

# Human smooth muscle cells cultured from atherosclerotic plaques and uninvolved vessel wall.

Eskin SG, Sybers HD, Lester JW, Navarro LT, Gotto AM Jr, DeBakey ME.

Smooth muscle cells (SMC) were cultured from atherosclerotic plaques and uninvolved arteries to determine if differences exist between growth characteristics or ultrastructure of the cultured cells. Eighteen aortic punch biopsies provided the uninvolved tissue, and 58 carotid plaques provided the atherosclerotic tissue. Eighty percent of the samples yielded viable cultured cells, which reached a maximum population doubling time during log phase growth of 72 h (seeding density =  $1.0 \times 10(4)$  cells/cm2, 2nd passage). Growth characteristics of both normal and plaquederived cells were the same in vitro. Growth rate declined with time in culture, and cell division ceased by the 5th or 6th passage. In culture, spindle shaped cells formed the "hill and valley" configuration typical of SMC. Plaque-derived SMC were ultrastructurally similar to SMC from uninvolved vessel wall. Proliferative potential did not vary with age or sex, with method of culture, or with whether the cells were plaque derived or not.